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Latency of Epstein-Barr Virus Is Stabilized by Antisense-Mediated Control of the Viral Immediate-Early Gene BZLF-1

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The ability of the Epstein-Barr virus (EBV) to avoid lytic replication and to establish a latent infection in B-lymphocytes is fundamental for its lifelong persistence and the pathogenesis of various EBV-associated diseases. The viral immediate-early gene BZLF-1 plays a key role for the induction of lytic replication and its activity is strictly regulated on different levels of gene expression. Recently, it was demonstrated that BZLF-1 is also controlled by a posttranscriptional mechanism. Transient synthesis of a mutated competitor RNA saturated this mechanism and caused both expression of the BZLF-1 protein and the induction of lytic viral replication. Using short overlapping fragments of the competitor, it is shown that this control acts on the unspliced primary transcript. RT-PCR demonstrated unspliced BZLF-1 RNA in latently infected B-lymphocytes in the absence of BZLF-1 protein. Due to the complementarity of the gene BZLF-1 and the latency-associated gene EBNA-1 on the opposite strand of the genome, we propose an antisense-mediated mechanism. RNase protection assays demonstrated transcripts in antisense orientation to the BZLF-1 transcript during latency, which comprise a comparable constellation to other herpesviruses. A combined RNAse protection/RT-PCR assay detected the double-stranded hybrid RNA, consisting of the unspliced BZLF-1 transcript and a noncoding intron of the EBNA-1 gene. Binding of BZLF-1 transcripts is suggested to be an important backup control mechanism in addition to transcriptional regulation, stabilizing latency and preventing inappropriate lytic viral replication in vivo. ***J. Med. Virol. 59:512–519, 1999.***

KEY WORDS: EBV; herpesvirus; B-lymphocyte; lytic; replication

INTRODUCTION

A characteristic feature of the Epstein-Barr virus (EBV) is its lifelong persistence in the host, an ability that is in various modifications shared with other members of the herpesvirus family. During insufficient immune surveillance, this persistence is the cause of various EBV-associated diseases [Okano, 1998]. Small resting B-lymphocytes have been shown to be targets for latent infection by EBV and to serve as a viral reservoir [Tierney et al., 1994; Miyashita et al., 1995; Decker et al., 1996]. In latently infected proliferating lymphoid cells, the viral EBNA-1 protein is invariably present, acting as a replication and transcription factor [Reisman et al., 1985; Ambinder et al., 1990]. Lytic replication of EBV was thought to happen in vivo predominantly in differentiating epithelial tissue [Becker et al., 1991; Young et al., 1991; Sandvej et al., 1992]. It was demonstrated recently that during primary infection with EBV (infectious mononucleosis, IM) [Anagnostopoulos et al., 1995; Prang et al., 1997] and in the absence of immunological control, the virus also replicates in the B-lymphocytes of the peripheral blood. However, the mechanisms deciding whether the virus in vivo remains latent or enters into lytic replication are unknown. In the lymphoid cells the lytic cycle of EBV is mainly triggered by the expression of the immediate-early gene BZLF-1 [Grogan et al., 1984; Countryman et al., 1987; Rooney et al., 1988; Taylor et al., 1989; Marschall et al., 1991; Bogedain et al., 1994], which downregulates several latent genes, including EBNA-1 [Kenney et al., 1989], and induces simultaneously the cascade of lytic cycle genes [Alfieri et al., 1991]. The activity of BZLF-1 itself is regulated by transcriptional control of the promoter [Kenney et al.,

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1995], by autocrine stimulation [Flemington et al., 1990], by posttranslational modifications and protein-protein interactions [Daibata et al. 1992; Katz et al., 1992; Kenney et al., 1992; Sinclair et al., 1992; Gutsch et al., 1993; Zhang et al., 1994].

Recently, it was demonstrated that the expression of BZLF-1 is further controlled on the posttranscriptional level [Prang et al., 1995]. Transient synthesis of a mutated competitor BZLF-1 RNA in latently infected lymphoid cells saturated this control mechanism and induced the expression of BZLF-1 and the lytic replication of the virus. Since the competitor RNA did not encode protein, these experiments indicated that in some of the latently infected cells the endogenous BZLF-1 gene was transcribed but further expression was blocked by a posttranscriptional control mechanism, unless the endogenous transcripts were released by the competitor RNA. The molecular basis for this control was unknown. However, since the lytic cycle gene BZLF-1 and the last intron of the latent cycle gene EBNA-1 are localized on opposite strands of the genome, an antisense-mediated mechanism was suggested. These transcripts in antisense orientation to lytic cycle genes during latency comprise a comparable constellation to other herpesviruses.

MATERIALS AND METHODS

Cell Lines and Transient Transfection Assays

BJAB is an EBV-negative, while P3HR1/16, Akata, and Raji are EBV-positive Burkitt's lymphoma (BL)-derived cell lines. B95-8 is a lymphoblastoid cell line, established from marmoset B-cells by immortalization with EBV in throat washings of a person with IM. B95-8–immortalized cells are known to contain about approximately 5% cells in lytic cycle. All cells were maintained in RPMI 1640 medium supplemented with 1 mM glutamine, 10% fetal serum, and 10 μg/ml gentamycin (Gibco, Eggenstein, Germany). Transfection of the lymphoid cells was performed by electroporation with a BioRad Gene Pulser essentially as described previously [Schwarzmann et al., 1994].

Recombinant Plasmids

The recombinant eucaryotic plasmids pCMVZmS, encoding the unspliced and mutated primary transcript of BZLF-1, and pCMVZ, encoding the BZLF-1 gene product Zta, have been described previously [Prang et al., 1995]. The cDNA of BZLF-1 was obtained by RT and PCR using the primers ON Anti 2 and 5′ Zta RT and inserted via 5′ protruding adenosine nucleotides into the PCR cloning vector pCR II (Invitrogen) to create the plasmid pCR Z-cDNA. To construct the plasmid pCMVZ-cDNA, encoding the spliced mRNA of BZLF-1, the cDNA was excised from pCR Z-cDNA and inserted into the plasmid pCMVZ via *Bst*X1 and *Sph*I to substitute the genomic DNA of BZLF-1. To create the plasmid pCMVZmS-cDNA, encoding the mutated and spliced mRNA of BZLF-1, a 223 bp *Pst*I/*Sph*I fragment, representing the exon 3 and part of exon 2, was excised from the plasmid pCR Z-cDNA and was inserted into the plasmid pCMVZmS via the *Pst*I/*Sph*I sites, creating an intermediate construct, which missed the complete genomic sequence of BZLF-1 downstream of the *Pst*I site of the mutated start codon. The missing fragment consisting of exon 1 and most of exon 2 was obtained by PCR from pCR Z-cDNA as template, using the primers ON *Pst*I and ON *Pst*II, and was inserted via *Pst*I into the intermediate construct.

To create the plasmids for the in vitro synthesis of short RNA fragments Z1 to Z7 of the unspliced and mutated RNA of BZLF-1, the fragments were obtained by PCR using the template pCMVZmS and the primers indicated in Table I and were inserted into the vector pGEM4Z via *Eco*RI and *Pst*I (pGEM4Z-) or via *Eco*RI and *Sph*I (pGEM4Z-δ5/6). For the eucaryotic vectors pCMV-δ1–7, the corresponding inserts were excised from the plasmids pGEM4Z-δ1–7 and inserted into pCMVZ via *Hin*dIII and *Eco*RI.

RNA Preparation

RNA was isolated of 1×10^6 cells from tissue culture by extraction in 1 ml RNAzol according to the manufacturer (WAK-Chemie, Bad Homburg, Germany). The RNA was resuspended in diethyl-pyrocarbonat–treated water with an final concentration of 40 U RNAsin and 5 mM DTT (Promega, Heidelberg, Germany).

RT-PCR Amplification Except RNase Protection PCR

RT-PCR assays were carried out with aliquots of RNA representing 2×10^6 cells per reaction. RNA samples were initially heated 5 min to 65°C removing secondary structures, followed by incubation at 37°C for 10 min allowing the 3′ primer-annealing for reverse transcription. RT-PCR was performed in a one-tube reaction with MMLV-reverse transcriptase (Perkin Elmer Cetus, Branchburg, NJ) according to the protocol of the manufacturer. The PCR products from 35 rounds of amplification were separated on 2% agarose gels, then Southern-transferred onto positively charged nylon membranes (Boehringer) and hybridized with oligonucleotids end-labeled with digoxigenin (Boehringer). For quality control of RNA samples, histone 3.3 RT-PCR was used according to Futscher et al. [1993].

In Vitro Transcription of RNA Probes and Labeling

Single-stranded RNA probes were generated by in vitro transcription using the Ribomax system (Promega) essentially according to the manufacturer.

Combined In Vitro Transcription-Translation

In vitro transcription and translation was done with the TNT/T7 system (Promega), essentially according to the instructions of the manufacturer.

RNase Protection Analysis

For RNase protection analysis, total RNA was purified by centrifugation through a CsCl cushion [Sam-

TABLE I. Sequences of Oligonucleotide Primer

Oligo	Sequence	Position
ON 2463	5′-CGGCATTTTCTGGAAGCCACCCGA-3′	102463–102486
ON 2764	5′-CTTGCCCCGGGGCTAACCAAGGAC-3′	102764–102741
ON 2573	5′-GTTGAAACAAGCCCCACCAT-3′	102573–102592
ON 2727	5′-CATTGGTGTTCCACAGCCTG-3′	102727–102708
ON NF 1	5′-GCTGCAGAGGCTCTGGCAGCACCG-3′	102987–103007
ON NF 2	5′-TGAATTCTGTGCTGTGGCCGGTGC-3′	103018–103002
ON NF 3	5′-CACTGCTGCAGCTGTTTGAACAGT-3′	102772–102795
ON NF 4	5′-GGAATTCACCTCAACCTGGAGACA-3′	102818–102801
ON NF 5	5′-ATCCGCTGCAGCCCCTCCATGAGC-3′	102591–102614
ON NF 6	5′-CGAATCCGGTAAGGAGGGGCTCAA-3′	102656–102639
ON NF 7	5′-GAGAATTCCGGGCCAAGTTTAAGC-3′	102465–102449
ON NF 8	5′-ACATGCATGCTTGGGCACATCTGC-3′	102276–102291
ON NF 9	5′-CGAATTCATCTGAAAATGACAGGC-3′	102326–102308
5′ Zta RT	5′-TTGCACCTTGCCGCCACCTTTG-3′	103194–103180
3′ Zta RT	5′-CGGCATTTTCTGGAAGCCACCCGA-3′	102463–102486
ON Anti 1	5′-GACAGGGAATTCACTAAACATTGC-3′	103213–103190
ON Anti 2	5′-AACCATGGCATGCACTTCAAAGAG-3′	102111–102134
ON Pst I	5′-AGCTGCAGTAATGAAACTCGAC-3′	103157–103136
ON Pst II	5′-TAGTGCTGCAGCAGTTTGCTTAA-3′	102431–102454

brook et al., 1989]. Thirty μg of total RNA, 30 μg yeast t-RNA, or 50 ng of single- or double-stranded control RNAs in 30 μg yeast t-RNA were dissolved in 20 μl hybridization buffer (50% formamide, 1-mM EDTA, 40 mM PIPES/NaOH, pH 6.4, 0.2 mM NaOAc). A total of 1×10^6 cpm of the radio-labeled RNA probe was added, secondary structures were removed by incubation for 5 min at 70°C, and the RNAs were annealed overnight at 40–50°C. The next day 270 μl RNase buffer (Promega) were added and single-stranded RNA was removed with 10-units RNase T1 and 1-unit RNase Q for 30 min at 37°C. Fifty μl proteinase K was added (1 mg/ml; 3.3% SDS in RNase buffer) and incubated for 1 hr at 37°C. The reaction was stopped with 25 μl stop buffer (10% SDS, 1 mg/ml yeast t-RNA) and the RNA was precipitated with ethanol. Finally, the RNA was dissolved in denaturing gel loading buffer (80% formamide, 1 mM EDTA, 0.1% bromphenolblue, 0.1% xylencyanol, 0.1% SDS), denatured by heat and electrophoresed in a 7% urea polyacrylamide sequencing gel in TBE buffer.

RNase Protection PCR

Thirty μg of total RNA or 50 ng of single- and double-stranded in vitro-transcribed control RNA in 30 μg yeast t-RNA were treated with 20–40 units of RNase T1 and 20–40 units of RNase One in RNase buffer (Promega). Incubation for 5 min at 99°C inactivated the RNase activity and simultaneously melted the double-stranded RNA. To prevent renaturation of the RNases and complete degradation of the RNA, the sample was shifted directly to 72°C and the single-stranded (hybrid-derived) RNAs were reverse-transcribed with Tth polymerase for 20 min and amplified by a standard PCR protocol according to the instructions of the manufacturer (Boehringer Mannheim). To increase the sensitivity of the assay, 10% of the product of the first PCR was amplified in a second nested PCR using Taq polymerase (35 cycles denaturation for 30 sec at 95°C, annealing for 45 sec at 55°C, and elongation for 60 sec at 72°C; finally, elongation for 10 min at 72°C).

RESULTS

The posttranscriptional block of BZLF-1 can be released with a full-length, unspliced competitor transcript of BZLF-1 but not with short overlapping fragments.

In order to identify the cis-acting elements on the BZLF-1 transcript, which might be involved in the posttranscriptional control, short overlapping fragments of the mutated unspliced primary transcript of BZLF-1 (Fig. 1) were transiently synthesized from eucaryotic expression plasmids in latently infected cells and tested whether these could induce expression of Zta and the lytic replication of EBV like the full-length (mutated) RNA.

To preclude activation by Zta, which might have been derived from the competitor RNAs, the AUG start codon was mutated and two stop codons were introduced downstream. Tested in in vitro transcription-translation assays (Fig. 2), the cDNA expression vector encoding the original spliced BZLF-1 mRNA yielded a protein with the authentic molecular weight of 32 kDa (lane 3). The vector encoding the original unspliced primary transcript gave rise to a truncated 27-kDa product (lane 1) due to a stop codon in frame within the first intron of BZLF-1. As expected, both the mutated unspliced transcript (lane 2) as well as the mutated mRNA (lane 4) were not translated at all, proving that the mutations were efficient in preventing the activation of the BZLF-1 protein. Furthermore, in contrast to the unmutated unspliced transcript, the mutated competitor did not induce expression of Zta after transient transfection in the EBV-negative cell line BJAB [Prang et al., 1995].

The transcription of the short RNAs and of the un-

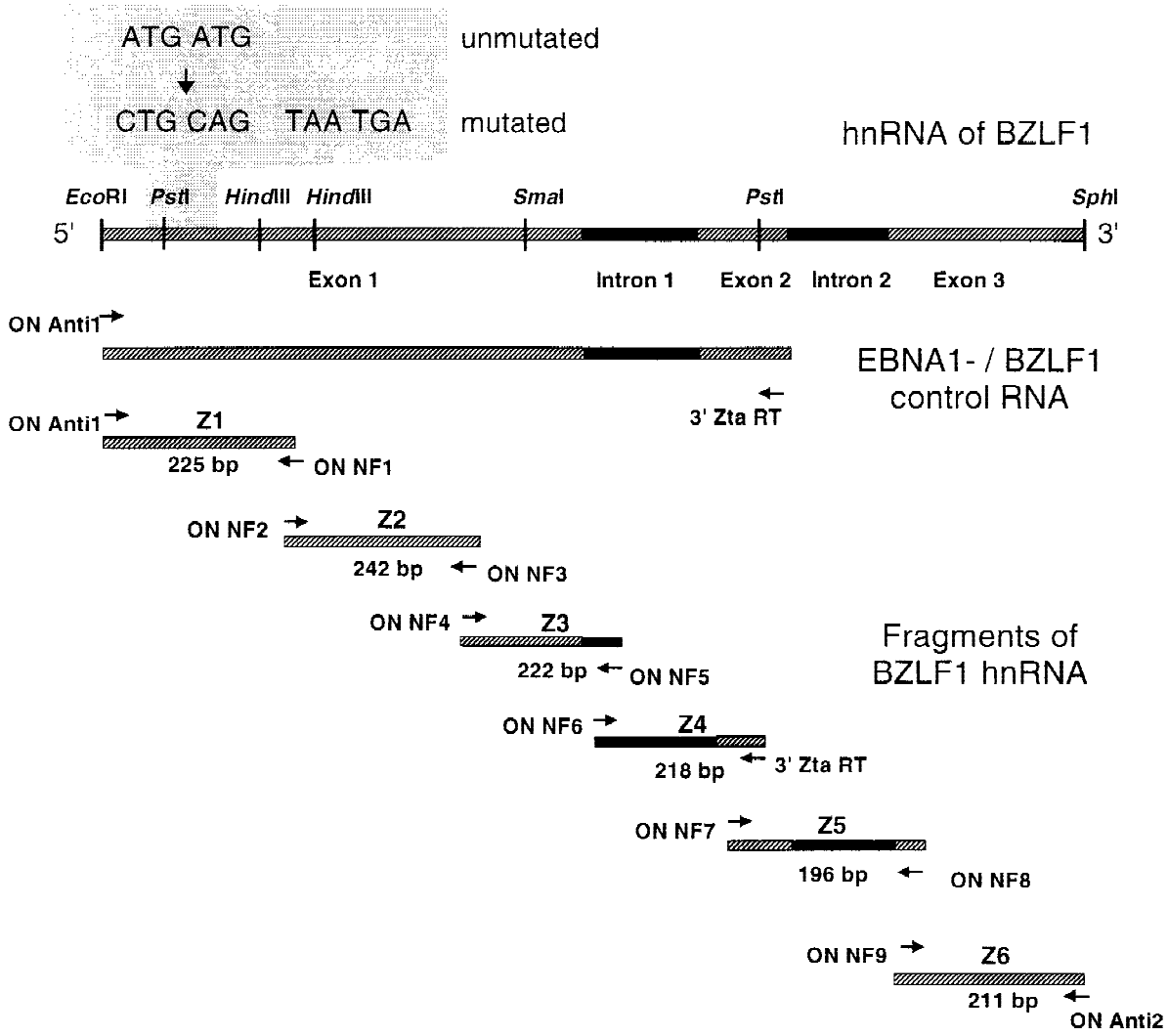

Fig. 1. Recombinant expression plasmids code for mutated and unmutated, spliced and unspliced full length transcripts of BZLF-1 and fragments thereof. The black boxes indicate intervening sequences, the hatched boxes represent exons. The primers used to construct the corresponding eucaryotic and procaryotic expression plasmids are indicated by short arrows.

spliced primary transcript was controlled by RT-PCR and efficient synthesis of all transcripts was seen at comparable levels (data not shown). Whereas the full-length mutated and unspliced competitor RNA induced expression of Zta (Fig. 3, lane 8), none of the short fragments did (Fig. 3, lanes 2 to 7). The spliced mRNA (Fig. 3, lane 9) induced the Zta protein, whereas mutated spliced competitor RNA (Fig. 3, lane 10) was unable to do so, which further confirmed that the introduced mutations rendered the competitor RNAs nonfunctional. CAT-mRNA (Fig. 3, lane 13) was nonfunctional as well. These transfection experiments demonstrated that only the full-length primary transcript, including the introns, was able to compete with the endogenous BZLF-1 transcript to interact with the regulatory mechanism and to release the endogenous BZLF-1 transcript during latency. Therefore, the post-transcriptional control mechanism acts on the unspliced primary transcript of BZLF-1.

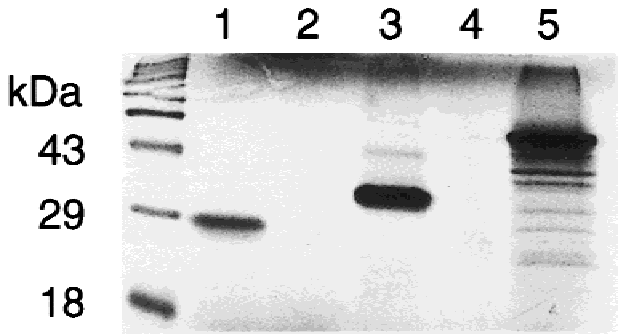

Fig. 2. The plasmid-encoded mutated transcripts of BZLF-1 were not translated. One μg of expression vector was used for in vitro transcription-translation in the presence of ^{35}S-labeled methionine. The reaction products were separated by SDS gel electrophoresis and visualized by fluorometry. **Lane 1**, pCMVZ′, yielding unspliced primary transcript; **lane 2**, pCMVZmS, yielding mutated unspliced primary transcript; **lane 3**, pCMVZ′cDNA, yielding spliced mRNA; **lane 4**, pCMVZmScDNA, yielding mutated spliced mRNA; **lane 5**, positive control (Luciferase).

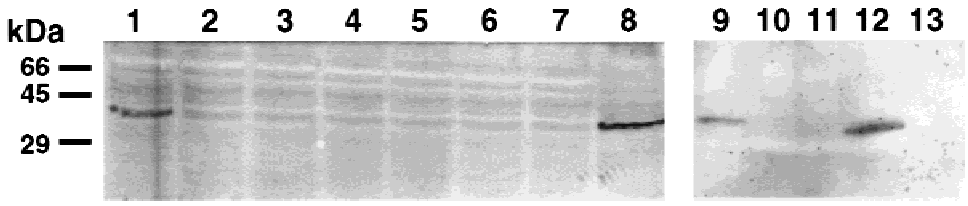

Fig. 3. The full-length unspliced competitor RNA of BZLF-1 but not short overlapping fragments released the posttranscriptional block of BZLF-1. Forty μg expression plasmid encoding either the fragments Z1 to Z6 of the BZLF-1 transcript (**lanes 2 to 7**), the full-length primary transcript (**lane 8**), spliced mRNA (**lane 9**), spliced mutated mRNA (**lane 10**), or CAT-mRNA (**lane 13**) were transiently transfected into the latently infected lymphoid cell line P3HR1. In **lane 11** only PBS without any plasmid was used. In **lanes 1 and 12** recombinant BZLF-1 protein, synthesized in *E. coli,* was applied as positive control. After 3 days cell extracts were prepared and the proteins were separated by SDS polyacrylamide gel electrophoresis. BZLF-1 protein was visualized by Western blotting and immunohistochemistry with a monoclonal antibody.

Unspliced transcripts of the immediate-early gene BZLF-1 in cultured latently infected cells indicate a block in RNA processing.

In order to detect transcripts of BZLF-1 in lymphoid cells infected latently in tissue culture (Fig. 4), reverse transcription (RT) and PCR were used with primers located in the first and the second exon of BZLF-1, bracketing the first intron (Table I). This allowed discrimination between spliced and unspliced RNA. Since the RNA was pretreated with DNase I, a contamination with genomic DNA was excluded (data not shown). With these primers we detected unspliced primary transcript of BZLF-1 (301 bp) in small amounts in 10 μg of total RNA of the cell lines P3HR1/16, Akata, Em-LCL (lanes 1, 2, and 3, respectively) and in large amounts in Raji cells (lane 4). An additional PCR product with a slightly larger molecular weight was attributed to nonspecific binding of the primers since the intensity of this band correlated inversely with the intensity of the expected product of 301 bp. With a subsequent nested PCR, with one of the primers spanning the exon-intron boundary, the unspliced transcript was clearly demonstrated in the four cell lines (lanes 7 to 10). These BZLF-1 transcripts indicated that in the tested, latently infected cells were at least a few cells that were permissive for initiating the lytic cycle. Detection of the primary unspliced transcript in addition to the spliced mRNA indicated that processing of the primary transcript was inefficient. With Western blotting, expression of the BZLF-1 protein was undetectable (data not shown), although RT-PCR detected spliced mRNA (177 bp) in P3HR1 and Akata cells (lanes 1 and 2). Therefore, either the number of permissive cells was too low and/or the amount of Zta was below the sensitivity of the assay, or the expression of the BZLF-1 gene was blocked at a posttranscriptional step.

Stable transcripts in antisense orientation to BZLF-1 can be detected in latently infected cells.

A hypothetical protein factor, responsible for the observed posttranscriptional control of BZLF-1, would have been expected to bind and to be titrated by at least one of the six overlapping BZLF-1–derived RNAs inducing expression of the Zta protein. Since this was not the case, we dismissed this possibility and focused on the idea of an antisense hybridization as a key mechanism. To find out whether stable RNAs in antisense orientation and complementary to the unspliced BZLF-1 transcript exist, RNase protection analysis were performed using short radio-labeled and EBNA-1 intron-specific RNA probes corresponding to fragments of the primary transcript of BZLF-1 (Fig. 5). The RNA probes 2 to 6, corresponding to position +102111 to +103018 of the standard B95-8 genome [Baer et al., 1984], were completely protected from degradation by RNase due to hybridization with complementary latent transcripts (lanes 6, 9, 12, 15, and 18). Only fragment 1 (positions +103213 to +102987) showed partial degradation of approximately 50 bases, indicating that this part of the transcript of BZLF-1 may not have been completely protected in vivo (lane 3). Negative controls with tRNA never showed any protection from degradation (lanes 5, 8, 11, 14, and 17).

In latently infected cells, the unspliced transcript of BZLF-1 forms a double-stranded RNA hybrid with the last 3′ intron of EBNA-1.

Since RT-PCR is the most sensitive method to detect nucleic acids, this technique was chosen to detect the double-stranded RNA hybrid, formed by the last 3′ intron of EBNA-1 and the primary transcript of BZLF-1 (Fig. 6). To perform this RT-PCR, it was critical to position the primers within the region of the postulated double-stranded hybrid. According to the RNase protection data, the most probable region for antisense hybridization was between positions 102111 and 103018 (Table I). To detect the BZLF-1–EBNA-1 hybrid, the primers ON2463 and ON2764 were located at positions 102463 and 102764, respectively, within the first and second exon, bracketing the intron, and thus allowing to discriminate between the amplification of the unspliced primary transcript and spliced mRNA. To amplify selectively the double-stranded hybrid, the total RNA from latently infected cells was treated with RNase T1 and RNase Q to remove single-stranded transcripts. In vitro-transcribed RNAs (positions 102486 to 103002) corresponding to the complete exon 1, the first intron and exon 2 of BZLF-1, which are complementary to EBNA-1, were used as single-

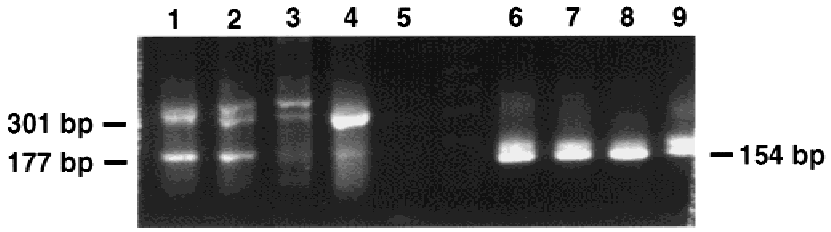

Fig. 4. Both spliced and unspliced transcripts of BZLF-1 were detected by RT-PCR in latently infected lymphoid cell lines. Ten μg of total RNA from different latently infected lymphoid cell lines was tested with RT-PCR (**lanes 1 to 4**), using primers within the first (ON 2764) and the second exon (ON 2463) of the BZLF-1 transcript. Amplification products of the unspliced primary transcript and the spliced mRNA were 301 bp and 177 bp in size, respectively. Nested PCR (**lanes 6 to 9**; primers ON 2573 and ON 2727) detected unspliced RNA in all samples yielding a product of 154 bp. Lanes 1 and 6, P3HR1/16; lanes 2 and 7, Akata; lanes 3 and 8, Em-LCL; lanes 4 and 9, Raji; **lane 5**, negative control without RNA.

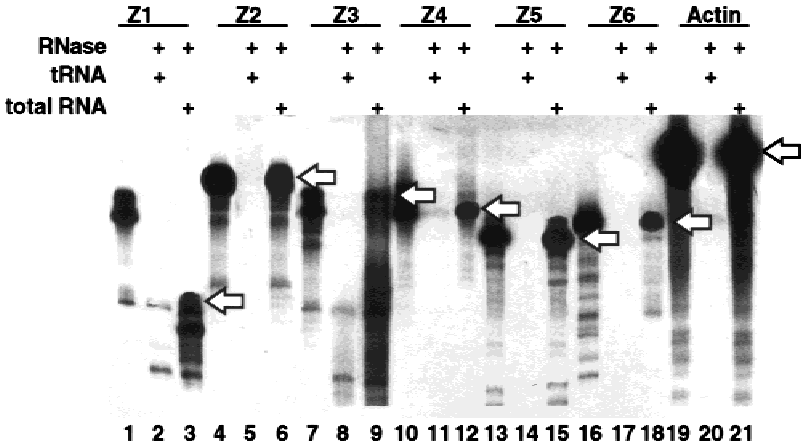

Fig. 5. RNA transcripts in antisense orientation to BZLF-1 were detected in latently infected lymphoid cells. With RNase protection assays RNA complementary to the primary transcript of BZLF-1 was detected with ^{32}P-labeled single-stranded overlapping RNA probes, derived from the unspliced BZLF-1 primary transcript (Fig. 1). A total of 1×10^6 cpm probe was hybridized overnight to 40 μg total RNA from latently infected P3HR1 cells and the next day RNase was added to remove any single-stranded RNA, including unhybridized probes. **Lanes 1, 4, 7, 10, 14, 16, and 19** show the labeled probes, without RNase**. Lanes 2, 5, 8, 11, 14, 17, and 20** show complete degradation of the probes when incubated with tRNA. **Lanes 3, 6, 9, 12, 15, 18, and 21** show the probes protected from RNase by hybridization to cellular RNA in antisense to BZLF-1.

stranded and double-stranded controls (Fig. 6A and B, lanes 1 to 6). Without RNase treatment, both the single-stranded and the double-stranded RNAs were detected by RT and PCR (lanes 1, 3, and 5). Treatment with RNase, however, abolished the signal of the single-stranded RNAs (lanes 2 and 4), demonstrating that these transcripts were not resistant to RNase due to secondary structures. The signal strength of the hybrid was nearly unaltered (lane 6). Testing the RNA of several cell lines, the RT-PCR (Fig. 6A) was too insensitive to detect a double-stranded RNA hybrid (lanes 8, 10, 12, 14, and 16). A subsequent nested PCR (Fig. 6B), Southern blotting, and hybridization to radio-labeled probes (Fig. 6C), however, detected BZLF-1 RNA, derived from a double-stranded RNA hybrid in the cell lines P3HR1/16, Raji, and Akata (lanes 8, 10, and 14). No hybrid was detected in the cell lines B95-8 and LCL-Em (lanes 12 and 16). The EBV-negative cell line BJAB was negative (lanes 18 and 19). Treatment of the RNA preparation with RNase in order to remove all single- and double-stranded RNA prior to the above-described RT-PCR protocol completely abolished any amplification signals showing that the signals were due to RNA and the preparation was free of DNA contamination. Accordingly, treatment with DNase had no effect (data not shown).

The observation that RNA probe 1 (positions 102111–103018) at the very 5′ end of the BZLF-1 transcript was not completely protected from degradation in RNase protection assays indicated that this part of the RNA might not have been complexed with the EBNA transcript. In accordance with this observation, a RNase protection/RT-PCR experiment comparable to that described above but using ON Anti 1 as 5′ primer at the most 5′ end of the transcript did not detect any protected RNA. This further confirmed the existence of the double-stranded hybrid RNA.

DISCUSSION

The location of the lytic cycle gene BZLF-1 complementary to an intron of the latent EBNA-1 gene theoretically allows posttranscriptional inhibition of BZLF-1, but does not affect expression of EBNA-1. A similar constellation of latency-associated transcripts in anti-

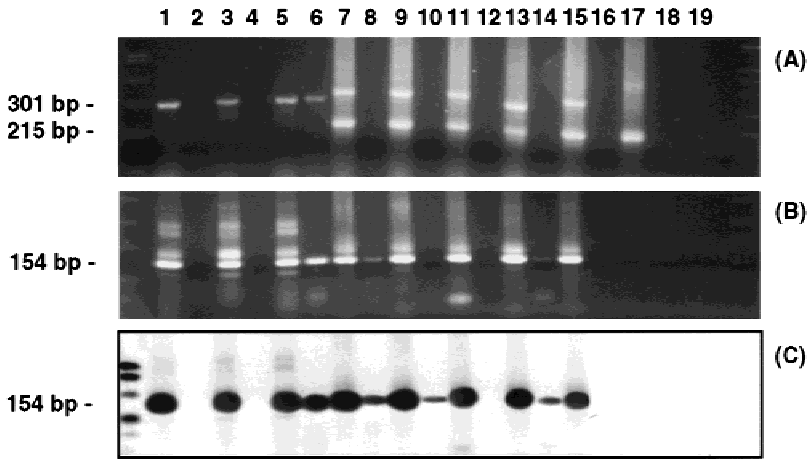

Fig. 6. RNase protection combined with RT-PCR detected double-stranded RNA consisting of the primary transcript of BZLF-1 and an intron of EBNA-1. Thirty μg of total RNA were treated with RNase T and RNase ONE to remove single-stranded RNA. The resistant double-stranded population was used for RT-PCR (**A**) and nested PCR (**B**) to detect complexed BZLF-1 transcripts. Additionally, the products from the nested PCR were Southern-blotted and hybridized to a specific probe (**C**). **Lanes 1 to 6** show controls with single-stranded BZLF-1 RNA (lanes 1, 2) and single-stranded EBNA-1 RNA (lanes 3, 4), which were not resistant to RNase (lanes 2, 4). Lanes 5 and 6 are double-stranded BZLF-1–EBNA-1 hybrids, which were protected from degradation (lane 6). **Lanes 7 and 8:** P3HR1; **lanes 9 and 10**: Raji; **lanes 11 and 12**: B95-8; **lanes 13 and 14**: Akata; **lanes 15 and 16**: LCL-Em; **lanes 17 and 18**: BJAB; **lane 19**: negative control/H_2O. In lanes 8, 10, 12, 14, 16, and 18, the RNA was treated with RNase T1 and RNase One prior the RT-PCR assay.

sense orientation to immediate-early genes of EBV has been described for a number of other herpesviruses. In the ganglia of mice and rabbits latently infected with HSV1, several transcripts (LATs) were described, which overlap the 3′ end of the immediate-early gene ICP0. In analogy to EBV, these are stable introns [Dobson et al., 1989; Farrell et al., 1991] derived by splicing from a larger transcript. In the trigeminal ganglia of pigs latently infected with pseudorabies virus (PRV) [Gutekunst et al., 1980; Rziha et al., 1997], latency-associated transcripts were described that are in antisense orientation, overlap with the 3′ end of the PRV immediate-early gene IE180, and may be derived from a larger RNA by splicing. For the bovine herpesvirus (BHV), which establishes latency in sensory and autonomic nerve ganglia [Homan et al., 1980], latency-associated transcription was reported in antisense orientation to an uncharacterized immediate-early gene [Rock et al., 1987]. Also in Marek's disease virus (MDV), two small RNAs were described, which map in antisense to the immediate-early gene (ICP4 homolog) of MDV and may be derived from a precursor RNA [Cantello et al., 1994]. However, so far a function of the LATs has been demonstrated only for HSV1 in the establishment of latency in neurones positively affecting the amount of reactivation from the latently infected ganglia due to an increase in the number of infected neurones [Farrell et al., 1991; Sawtell et al., 1992]. It was demonstrated above that a double-stranded hybrid RNA was the basis of a posttranscriptional regulation mechanism, and functional data were described that this mechanism functions in vivo and controls the activity of a gene and the complete lytic replication of a virus.

The molecular consequences of this antisense hybridization are not clear. The detection of an unspliced primary transcript of BZLF-1 and the observation that the appearance of the spliced mRNA correlated with the induction of BZLF-1 [Prang et al., 1995] suggested a block in the processing of the primary transcript. Accordingly, the RNase protection PCR experiments demonstrated unspliced BZLF-1 RNA in the double-stranded hybrids. Formation of a hybrid may sterically interfere with either initiation or progression of translation. This seems not to be the case for EBV, since spliced and mutated competitor RNA was not able to release repression. Double-stranded RNA may be degraded by free RNases, RNases associated with ribosomes [Wagner et al., 1990; Hentze, 1991] or by unwindases [Nellen et al., 1993]. Modification of RNA for subsequent degradation has been discussed for the regulation of the gene encoding the fibroblast growth factor in Xenopus oocytes [Kimelman et al., 1989].

In conclusion, the control of transcription of the immediate-early gene BZLF-1 is undoubtedly the first and probably the most important control of the lytic replication of EBV. Due to autocrine stimulation, small variations of the transcriptional activity of the gene could destabilize latency and result in inappropriate replication of the virus. The posttranscriptional control mechanism, based on antisense hybridization of BZLF-1 and EBNA-1 RNAs, provides a backup control mechanism to stabilize latency. By definition, the hybrid requires initiation of the lytic cycle. Therefore, the double-stranded hybrid RNA will only be detected in a small number of latently infected cells, since most others are strictly latent due to transcriptional repression. To release this control and to induce lytic replication, the ratio of transcripts of both genes might influence the expression of BZLF-1. Either the activity of the promoter of the BZLF-1 could be enhanced to override posttranscriptional control or the EBNA promoter may be downregulated to shift the balance in favor of lytic replication.

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